<u>Fusarium</u> <u>moniliforme</u> Sheld. Association with Species of Orchids

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Abstract. The fungal species <u>Fusarium</u> <u>moniliforme</u> Sheld. was
repeatedly isolated in nature from orchid species <u>Cypripedium</u>
<u>calceolus</u> L. and <u>C</u>. <u>reginae</u> Walt. A symbiotic relationship was
found between the fungus and orchids native to Michigan. Induced
fungal pathogenicity was obtained with <u>F</u>. <u>moniliforme</u> in the orchid
<u>Zygopetalum</u> <u>machaeii</u>.

Introduction

Over 50 orchid species are found native to Michigan (11,17).
Occasionally good populations of selected species occur in undis-
turbed habitats. From the Michigan species, <u>Cypripedium</u> <u>reginae</u>
Walt and <u>C</u>. <u>calceolus</u> L. were selected for mycological investigat-
ions. The habitat of <u>C</u>. <u>calceolus</u> is cosmopolitan and it can be
found in tamarack - black spruce bogs, in heavily wooded sphagnum
swamps, in thickets along streams, in bushy meadows, on sandy
swampy lake shores, and in damp meadows, fields and roadsides.
<u>Cypripedium</u> <u>reginae</u> is slightly more restricted to such areas as
bogs, heavily wooded swamps, swampy lake shores, and in areas where
sphagnum grows well.

Initial investigations examined selected orchid plants for the
presence of fungi. Repeated isolations of <u>Fusarium</u> <u>moniliforme</u>
Sheld. were obtained from various parts of mature orchid plants
growing in nature. Orchids are known to possess chemical and morph-
ological qualities which control or hinder fungal growth. Phytoalexins
keep invading fungal species under control, creating symbiotic,
commensalistic or slightly parasitic relationships (1,3,4).

Various inoculations of <u>Fusarium</u> conidia, originally isolated
from field material, were made on domestic orchids. The fungal
associations with the orchids created by the inoculations were then
examined. Studies were designed to identify the relationship of
<u>Fusarium</u> sp. to domestic and native orchids of Michigan.

Literature Review

Regardless of differences in morphological, anatomical, or
physiological characteristics, all orchids at some phase in their
life cycle are associated with certain fungi. Hyphal filaments
serve as absorbing organs within cortical cells of orchid roots (31,
32,33). This orchid - fungal association may be advantageous, neutral,
or disadvantageous and parasitic (2). Some species of fungi are
found throughout the orchid plant, but mycorrhizae create a symbiotic
relationship in the host root system (10,34,38,41). In a mycorrhizal
relationship, reciprocal movements of substances between the orchid
and the fungus occurs (3,4,35). Little evidence either supporting or
discounting the specificity of the host - endophytic relationship has
been established, but it appears that certain fungal species seem to

be more effective with select taxonomic groups of orchids (29,31,34).

According to Marx (38), the micorrhizae of orchids have an endo-mycorrhizal association. Hyphae of the symbiont penetrate the cells and establish close contact with the cytoplasm. Endomycorrhizal fungi have the ability to degrade cellulosic cell walls, but ecto-mycorrhizal fungi do not have this ability and are limited to util-izing a few simple carbohydrates (41). Apparently seedlings require association with specific fungi soon after seed germination. Fungi infect orchid embryos in the early protocorm stage. Without this infection, embryonic growth continues only by an artificial supply of sugars, vitamins, as well as mineral nutrients (19,42,47,48). Nutrients are first absorbed by the fungal hyphae and then released into the host tissue. Fungal hyphae are digested in the host cells, which is the transfer mechanism of nutrients from fungus to host (15,28,33).

The orchid host has many methods of controlling the fungal symbiont. It is clear that the host - endophyte complex must main-tain factors or conditions that bring enzyme activity of the fungus under control. If this control is not attained, a pathogenic asso-ciation could result. The controls of the enzyme activity of the fungus are due to the interaction of metabolic processes of the endophyte and the host. The fungus is able to break down soil cell-ulose into nutritive compounds that can be absorbed by the fungus and then in turn by the host (47,48). The absence of invading hyphae in various parts of terrestrial orchids has suggested that orchids possess a mechanism of resistance to, or a substance that is toxic to, mycorrhizal fungi (32). This fungistatic control compound is secreted only by living cells due to the activity of the fungus upon them. Structurally, the chemical is a benzene soluble fungistatic compound with a molecular weight of 256. The chemical was named Orchinol (25,26). It was the first of several compounds to be dis-covered that were produced by orchids after infection by fungi and which were classified as phytoalexins (1,3,13,14,21,22,24,31,40,49). Phytoalexins are described as plant antibiotics which are inhibitory to microorganisms attacking plants. The compounds arise from meta-bolic interactions of hosts and parasites (43). Other compounds discovered following studies on phytoalexins were Hircinol and Lor-oglossol which had a wide spectrum of activity on fungi, however, their modes of action were not specific against only mycorrhizal fungi (20,21).

The formation of phytoalexin is induced in the plant by diverse fungi. The fungal types include obligate and facultative parasites, specifically adapted pathogens to the plant, and phytopathogenic fungi not infecting the given plant species (40). However, all fungi do not necessarily bring about phytoalexin formation. Orchinol was not form-ed when common saprophytic and semiparasitic soil fungi were used for the infection of orchids (25,26). Phytoalexin formation occurs in all host plant organs. A plant is resistant to a parasite if the concen-tration of phytoalexin is high enough to inhibit the growth of a parasite. The orchid becomes a susceptible host if the phytoalexin concentration is insufficient to inhibit the pathogen (40). It is not known if the effect of these substances allows certain mycorr-hizal fungi to exist in roots and at the same time inhibit the asso-

ciation of other fungal species. This could explain the selectivity of fungal species to orchid symbiosis (32).

Hyphae invading orchid tissues are surrounded by a membrane that is close to the fungal wall or separated from the host tissue by a distinct layer. This membrane is considered to be of host origin while the metabolic transfer is from fungus to host (13). Intracellular hyphae did not penetrate the plasmalemma of the orchid cell (29).

Not all fungal relationships with orchids are beneficial, and a pathogenic state can exist between the fungus and its host. The difference between a mycorrhizal fungus and a root pathogen may not be great (48). The occurrences of fungal pathogenicity in orchids are few, but there are examples of serious pathogenic associations (9,16,18,39). Certain species of fungi can cause severe root rot in various genera of orchids (2,36,46). Bud rots and leaf stains are other diseases caused by fungi and are characterized by discolored spots or patches on the leaves, stems, and fruit which are followed by death or rotting of these organs (5). The association of F. moniliforme with domestic and wild orchids was examined to determine what relationship was established between the orchids and the fungal isolate.

Materials and Methods

Two orchid species, Cypripedium reginae Walt. and Cypripedium calceolus L. were selected for study. The collection site was Kalkaska County, Michigan. The habitat consisted of a balsam - cedar forest opening onto a bog. Mature plants, soil samples, and orchid leaf litter were obtained at the collection sites then taken to the laboratory.

One inch sections of orchid leaves from living plants were surface sterilized with 70% ETOH 5 seconds and 5% Chlorox bleach 15 minutes before placing in the moist petri chamber. Sterile distilled H_2O was periodically added to the petri plates to prevent drying of the orchid material. Pure cultures of the fungi isolated from the incubated material were maintained on Potato Dextrose Agar (PDA) and identified according to the literature (6,7,8,12,23,24,27,37,45,46).

Serial dilutions of soil suspensions were plated on PDA and incubated at room temperature. Orchid leaf litter collected in nature was placed in moist petri dish chambers and incubated. Fungal isolates were identified and maintained in pure culture.

Mature hybrid orchid plants were obtained from the collections of Ilgenfritz Nurseries, Monroe, and Black River Orchids, Grand Haven. Hybrids selected included Cattleya sp., Cypripedium sp., Cymbidium sp., Dendrobium sp., Diacattleya sp., Epicattleya sp., Epilaeliocattleya sp., Laeliocattleya sp., Oncidium sp., Paphiopetalum sp., Phalaenopsis sp., Sophrolaeliocattleya sp., and Zygopetalum sp.

Hybrid orchid leaves were incubated with fungal colonies on agar by placing the colony surface down on the leaf blade. After 3 weeks incubation leaf sections containing the colony inocula were removed from the plant, fixed in FAA, dehydrated in an alcohol series, and embedded in Tissuemat. Material was sectioned at 8-10 u then placed on slides controled with Haupts adhesive (1 g gelation, 2 g phenol,

15 ml glycerine to 30 ml distilled H$_2$O) and flooded with 4% formalin.
Slides were drained and air dried one week before staining. Sect-
ioned plant material invaded with fungi was stained with a modified
Conants quadruple stain in a xylene alcohol dehydration, safranin in
30% ETOH, orange gold and fast green in clove oil, with a Canada
balsam mounting.

Excised Cypripedium roots, leaf sections, and stems were
obtained from living plants in nature, surface sterilized then
placed on PDA, Knudson's Orchid Agar, and Noble Agar for growth and
isolation of fungi associated with the plant tissue.

Results

Leaf litter and soil obtained adjacent to Cypripedium species in
nature contained numerous fungi and bacteria. Fusarium moniliforme
was the dominant fungal species most frequently found in each coll-
ection. Repeatedly F. moniliforme was isolated from living Cypri-
pedium leaves that were surface sterilized and placed in incubation
chambers. A suspension of F. moniliforme conidia inoculated into
various hybrid orchids caused blackening of the host tissue at the
inoculation site with no further fungal involvement.

One orchid, Zygopetalum machaeii served as a suitable host
plant for fungal invasion from agar block transfers of F. moniliforme
cultures to the orchid leaves. Other orchids that proved negative
with this method included Cymbidium balkis, Dendrobium rididum, Dia-
cattleya sp., Epicattleya sp., Epilaeliocattleya sp., Laeliocattleya
sp., Oncidium ampliatum, Paphiopetalum callosum, Phalaenopsis sp.,
and Sophrolaeliocattleya sp. With Zygopetalum sp., a darkened area
appeared similar to the inoculations on the other plants, and in
addition, Fusarium established a vigorous colony on the leaf surface
independent of the agar block.

Sectioned Z. machaeii leaf tissue demonstrated a definite fungal
association. Sclerotia were found in abundance in the leaf tissue.
Most sclerotia were round or kidney shaped with a defined dark colored
outer rind enclosing a medulla of densely packed hyphae lacking any
consistent orientation. Some microsclerotia were observed contain-
ing few cells without an outer rind. In one case the whole end of
the leaf had been taken over by a sclerotial cap. The sclerotial
rind contained coalesced hyphal segments with thick, agglutinated,
dark colored walls. Agglutinated segments were also found within the
medulla interior. Internal consolidation consisted of intercalary
expansion and septation associated with hyphal anastomoses. Reserve
materials accumulate as the wall thickened.

The palisade and mesophyll areas of the leaf were heavily
invaded with mycelia. The upper and lower epidermis appeared desic-
cated and completely replaced by hyphae. Macroconidia were common
near a sclerotium. Aerial hyphae on the abaxial sides of the leaves
were abundant, and remnants of the leaf cells could be seen surround-
ing the rind of the sclerotium on the adaxial side.

In several leaf sections of Z. machaeii, many stromatic pustules
were found. These pustules served as perithecial initials or imma-
ture sporodochia. Hyphae were dominant throughout the entire leaf
section, and the epidermis on both adaxial and abaxial sides was
lifted or sloughed off near the stromatic pustules (Fig.l). Pustules

found directly over a vascular bundle had a tendency to invade the vascular bundle but apparently did not disrupt the function of the tissue. Macroconidia were very abundant and aerial hyphae were again found in large amounts. Hyphal penetration appeared to be both intercellular and intracellular and some cells were completely packed with hyphal strands. The pustules appeared heavily packed with pseudoparenchymatous material with small amounts of agglutin-ated cells as found in the sclerotium.

Sectioned Cypripedium roots appeared heavily infected with F. moniliforme. The epidermis and inner cortical cells were dense with hyphae and conidia (Fig.2). Inner cortical cells also contained loosely packed hyphal strands with macroconidial formation on phialides. Large stroma were also observed which appeared dark in areas and contained large isodiametric cells with slightly thickened walls. Aerial hyphae were found in abundance with macroconidia.

Cypripedium sp. seeds collected in nature were surface steril-ized and shaken vigorously in sodium hypochlorite solution and inoculated on orchid agar plates. The seeds prior to inoculation were stored 6 months at room temperature and 6 months in a freezer to induce the overwinter cycle. Soon after incubation the seeds supported fungal growth. The organism was identified as pure cultures of F. moniliforme.

Cypripedium roots were obtained from potted plants transported to the laboratory from the original collecting site. Surface sterilized roots placed on PDA, orchid agar, or nutrient agar supp-orted abundant mycelial growth within 5 to 7 days incubation at room temperature. The predominant fungal species again was F. moniliforme.

Fusarium moniliforme form microconidia in chains or on poly-phialides, spindle to ovoid in shape. The macroconidia are slender with thin walls, commonly three septate, having an appearance of quarter moons. The cultures range in coloration from brownish-white to orange-cinnamon with a stroma white to deep violet. No chlamydo-spores are formed, however, large sclerotial beds occur over the host or substrate. Growth on PDA is initially filmy, colorless, and rapid. The reverse colony surface becomes typically deep violet but sometimes lilac or cream. Aerial mycelium is generally dense with a felt texture.

Discussion

It was determined by this study that an association does exist between Cypripedium reginae, C. calceolus and Fusarium moniliforme. The complete nature of this association remains unknown. It is highly probable that a symbiotic relationship developed between the opportunistic fungus F. moniliforme and the Cypripedium species, and that a mycorrhizal situation exists within the roots of these orchids.

The greatest Fusarium growth was associated with the roots of the Cypripedium species. This is the area in the orchid plant where the concentration of phytoalexins would be at the lowest level there-fore accounting for the profuseness of Fusarium. When pure cultures of Fusarium moniliforme were reintroduced onto leaves, stems, or other tissues of living Cypripedium orchids there was no response and the fungus eventually died after the small agar block on which

it was growing was depleted. When various commercial orchids were
used for inoculation purposes, in all but one example, Zygopetalum
machaeii var. Lee, there was no outward response noted. Only a
browning effect caused by the apparent scar tissue produced by the
orchid in response to the scalpel cuts. Microscopic examination of
embedded tissue of this brown area revealed no fungal hyphae or
fungal remnants, and the underlying orchid cells appeared normal.
A lesion appeared on Zygopetalum after Fusarium inoculation. The
Fusarium eventually grew entirely through the leaf and appeared
healthy and vigorous. Stromatic pustule lesions were observed on
both the adaxial and abaxial sides of the leaf. These lesions may
be perithecial initials or they may develop into true sporodochia
(12). Scattered lesions on the Zygopetalum leaves appeared craterous
with the centers sunken and containing a large amount of hyphal
material situated around the outside of these pustule craters. Macro-
conidia were apparent on the surface around the pustules, and agglu-
tinated hyphae were also present. Host leaf cells were partially
filled with hyphae. No vascular tissue of the Zygopetalum orchid
plants was affected by the fusarial growth. Even in cases where
the fungus was growing adjacent to the vascular bundles there was
no evidence of plugging or any disarray of xylem vessels. In most
fusarial infections of other plants the vascular system is attacked
and the fungal presence is observed. Cells immediately beneath the
stromatic pustule appear to be normal without the appearance of
being crushed or mutilated even though many cells are filled with
fungal hyphae. The infected area was localized to the section of
leaf where initial infection had taken place. There was no evidence
of wilt as if the vascular system was being invaded and only the
local lesions were apparent. Sclerotia formed on the ends and sides
of the Zygopetalum leaves and apparently caused malformation of
host cells. Epidermal areas as well as cortical cells were completely
dominated by the sclerotia.

In the Cypripedium roots no stromatic pustules were formed or
detected upon examination of the infected tissue. The emerging
Fusarium fungus was allowed to completely envelop the root in the
incubation chambers before killing-fixing. When sectioned, infected
roots were found to be free of any outward breaks in the root epi-
dermis caused by the fungus, however, the roots were completely
infiltrated with Fusarium. It was quite evident that the fusarial
growth developed within the root before it emerged at the surface.
The epidermal cells were packed with the fungus, but there was
little disruption of cell walls.

The relationship of fungi with orchids whether pathogenic,
saprophytic, opportunistic or symbiotic is of a highly complex nature.
Hardy orchid species survive by the establishment of an association
with the fungal invader. Cypripedium species are similar to other
orchid genera in that associations exist with a fungus which are
necessary for the survival of the orchid. Fusarium moniliforme was
determined by this study to have a definite association with Cypri-
pedium reginae and C. calceolus. No lesions occurred in Cypripedium
in spite of the dominance of F. moniliforme in healthy plant material,
however, a commercial orchid developed lesions by the invading fungus.
Fusarium moniliforme and Cypripedium species in Michigan appear to be

symbiotic while F. moniliforme with Zygopetalum machaeii is parasitic.

Summary

    Fusarium _moniliforme_ Sheld. was isolated repeatedly in nature from Cypripedium reginae and Cypripedium calceolus, orchids native to Michigan. _Fusarium_ _moniliforme_ introduced onto various cultured orchid species produced no pathogenicity in all but one species. Stromatic pustules developed on upper and lower leaf surfaces of Zygopetalum machaeii, and macroconidia were found in abundance. Sclerotia formed on Zypopetalum leaf tissue and they were composed of well developed outer rinds surrounding the medullae of agglutinated hyphae. The leaf was also desiccated and malformed by the fungus. _Fusarium_ _moniliforme_ was symbiotic to C. reginae and C. calceolus, and pathogenic to Z. machaeii.

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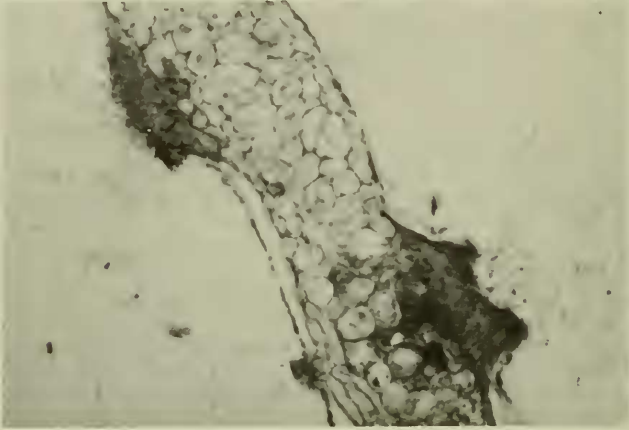

Figure 1. Stromatic pustules of Fusarium moniliforme on the adaxial and abaxial surfaces of Zygopetalum machaeii leaf. x 390.
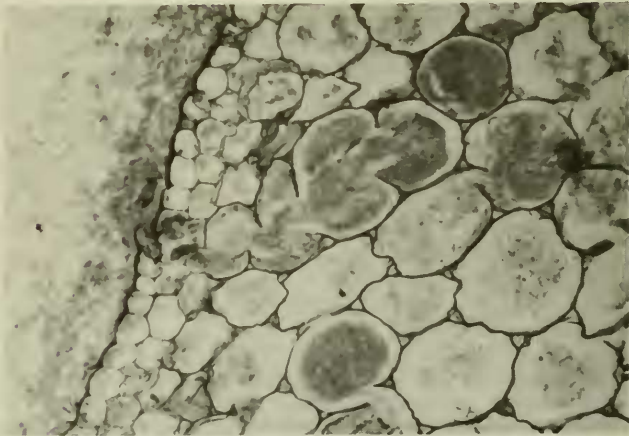

Figure 2. Cypripedium root with a dense lateral development of Fusarium moniliforme in epidermal and cortical cells in addition to loosely organized mycelial formation randomly found in host tissue and a dense growth on the host leaf surface.   x 390.